Vaccine 33 (2015) 437–445

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The *Plasmodium berghei* sexual stage antigen PSOP12 induces anti-malarial transmission blocking immunity both in vivo and in vitro

K.A. Sala[a,1], H. Nishiura[b,1], L.M. Upton[a], S.E. Zakutansky[a], M.J. Delves[a], M. Iyori[b], M. Mizutani[b], R.E. Sinden[a,c], S. Yoshida[b], A.M. Blagborough[a,*]

[a] Department of Life Sciences, Sir Alexander Fleming Building, Imperial College London, Imperial College Road, South Kensington, London SW7 2AZ, UK
[b] Laboratory of Vaccinology and Applied Immunology, School of Pharmacy, Kanazawa University, Ishikawa, Japan
[c] Jenner Institute, The University of Oxford, Roosevelt Road, Oxford OX9 2PP, UK

ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form
19 November 2014
Accepted 20 November 2014
Available online 30 November 2014

Keywords:
Plasmodium
Transmission-blocking
PSOP12
Gamete
Ookinete
Baculovirus

ABSTRACT

Anti-malarial transmission-blocking vaccines (TBVs) aim to inhibit the transmission of *Plasmodium* from humans to mosquitoes by targeting the sexual/ookinete stages of the parasite. Successful use of such interventions will subsequently result in reduced cases of malarial infection within a human population, leading to local elimination. There are currently only five lead TBV candidates under examination. There is a consequent need to identify novel antigens to allow the formulation of new potent TBVs. Here we describe the design and evaluation of a potential TBV (BDES-PbPSOP12) targeting *Plasmodium berghei* PSOP12 based on the baculovirus dual expression system (BDES), enabling expression of antigens on the surface of viral particles and within infected mammalian cells. In silico studies have previously suggested that PSOP12 (Putative Secreted Ookinete Protein 12) is expressed within the sexual stages of the parasite (gametocytes, gametes and ookinetes), and is a member of the previously characterized 6-Cys family of plasmodial proteins. We demonstrate that PSOP12 is expressed within the sexual/ookinete forms of the parasite, and that sera obtained from mice immunized with BDES-PbPSOP12 can recognize the surface of the male and female gametes, and the ookinete stages of the parasite. Immunization of mice with BDES-PbPSOP12 confers modest but significant transmission-blocking activity in vivo by active immunization (53.1% reduction in oocyst intensity, 10.9% reduction in oocyst prevalence). Further assessment of transmission-blocking potency ex vivo shows a dose-dependent response, with up to a 76.4% reduction in intensity and a 47.2% reduction in prevalence observed. Our data indicates that PSOP12 in *Plasmodium* spp. could be a potential new TBV target candidate, and that further experimentation to examine the protein within human malaria parasites would be logical.

1. Introduction

Malaria is a global disease of man caused by parasites of the genus *Plasmodium*, which are transmitted exclusively by *Anopheles* mosquitoes. Global morbidity and mortality have been substantially reduced over recent years [1], due to the use of interventions such as long-lasting insecticidal nets and increased access to care. Despite this, there are currently an estimated 219 million human cases annually, causing 627,000 deaths per year, with the majority of disease burden in African children under the age of 5 [1]. The tools currently available to combat the disease are insufficient to interrupt transmission in areas of higher endemicity [2] and there remains growing concern about the spread of insecticide and parasite drug resistance [3].

It is now widely agreed that to achieve eradication, it is necessary to use interventions that inhibit the transmission of parasites from humans to mosquitoes. Successful use of such interventions, either alone, or in combination with other anti-malarial measures, will subsequently result in reduced cases of malarial infection within an endemic population leading to local elimination. A potential manner of achieving this is by targeting *Plasmodium* using transmission-blocking vaccines (TBVs) against the sexual stages of the parasite. TBVs have a number of unique biological advantages

* Corresponding author. Tel.: +44 0 207594 5350.
E-mail address: andrew.blagborough@imperial.ac.uk (A.M. Blagborough).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.vaccine.2014.11.038

over vaccines targeting other stages of the *Plasmodium* lifecycle; these include low numbers of the target parasite stages, a comparatively long vulnerable extracellular period, and reduced antigenic polymorphism [4]. It has been previously demonstrated in numerous studies that transmission-blocking antibodies can successfully inhibit transmission from vertebrate to mosquito. Both fertilization and ookinete formation within the midgut can be inhibited by the presence of vaccine-induced antibodies within the mosquito blood meal. Fertilization can be blocked through agglutination, steric hindrance, or complement-mediated lysis of gametes [5,6], whereas ookinete development can be inhibited or fully prevented by complement-mediated lysis, melanization, antibody-dependent phagocytosis or by blocking recognition of the midgut epithelial cells [7,8]. In the laboratory transmission-blocking antibodies can result in profound and often total reduction s in parasite transmission to the mosquito vector.

A wide range of different immunogenic targets that potentially induce transmission-blocking activity in *Plasmodium* have been investigated over the last three decades. Five parasite-derived proteins are currently considered to be lead TBV candidates; P25 and P28 (both expressed on the surface of zygotes and ookinetes), P48/45, P230 and HAP2. P48/45 is expressed by gametocytes and is present on the surface of male gametes [9,10]. The protein plays a key role in parasite fertilization, and targeted disruption of the gene affects the male gamete's ability to attach to female gametes. [11]. Anti-P48/45 antibodies are found in human serum from endemic areas, and their abundance correlates with transmission-blocking activity [12–14]. Transmission-blocking induced by recombinant protein-derived P48/45 antibodies is significantly dependent on the recognition of conformation-dependent epitopes [15]. P230, also present on the surface of the gamete, is a promising TB immunogen. Antibodies against P230 inhibit or prevent oocyst development [16,17], and have been demonstrated to lyse gametes in a complement-dependent manner. Previous studies have suggested that P48/45 and P230 form a complex at the surface of gametocytes and gametes [18,19]. More recently, HAP2, a conserved male protein, located on the surface of the gamete plasma membrane has demonstrated potent transmission-blocking efficacy, in both the rodent malaria parasite *Plasmodium berghei* [20], and subsequently, the human malaria parasite *P. falciparum* [21].

Despite the large number of proteins that have been previously screened for the ability to initiate a transmission-blocking response, the pool of parasite-derived TBV immunogens for which compelling evidence exists is disappointingly small. Only five parasite-derived proteins are currently considered to be lead candidates, a number that is unacceptably small when considering the future development pipeline of TBVs. As a result, there is a real need to identify novel potential antigens to allow the formulation of new potent TBVs in the future and to permit the potential combination of multiple immunogens in new anti-malarial vaccine formulations [22]. Two of the five currently proven, potent transmission-blocking immunogens are present on the surface of the ookinete [23,24], whereas the other three have conformed localization on the surface of the male gamete [18,19,25]. Previous proteomic experimentation has resulted in the completion of a proteome of the *P. berghei* male gamete, with subsequent in-depth bioinformatics analysis [27]. Automated *in silico* analysis for cellular location and function was carried out, using a raft of 17 individual prediction programmes [27], to generate consensus predictions for each individual protein identified within the proteome. From 624 proteins in total, 506 were successfully assigned a putative cellular location/function, subject to experimental confirmation. Within this group, 23 molecules were classified as both "hypothetical" (i.e. with no detectable homologues or predictable function) and "putative surface molecules" (i.e. located within, or associated with, the plasma membrane). Given these predicted characteristics (novel and on the surface of male gametes), it is logical to investigate the ability of these proteins to induce a potent transmission-blocking response and potentially act as targets for novel TBVs. Amongst these proteins was the previously identified parasite protein, Putative Secreted Ookinete Protein 12 (PSOP12/PBANKA_111340). PSOP12, like the lead TBV candidates P48/45 and P230, is a putative member of the well-characterized 6-Cys family of proteins, of which 10 members have been characterized [19], with a further 4 "6-Cys family associated" proteins (including PSOP12) identified later [28]. Members of the family are characterized by domains of approximately 120 amino acids in size, containing six positionally conserved cysteines. Most members of the family are expressed in a stage-specific manner stages (gametocytes, sporozoites or merozoites) [19]. Five 6-Cys genes are transcribed in gametocytes, and multiple studies [11–18] have successfully demonstrated that the members of this protein family play a role in the process of gamete recognition and fertilization.

PSOP12 has been previously identified in a reverse genetics screen and medium-throughput knockout study in *P. berghei*, and was demonstrated as expressed in the ookinete [29]. Expression of the protein is predicted in both the *P. berghei* gametocyte and ookinete [30]. A signal peptide is located at amino acids 1–24, whereas our in depth bioinformatics consensus analysis [27], and other recent studies [28] have predicted a potential single 6-Cys domain at the C-terminal of the protein. The presence of a 6-Cys domain in the PSOP12 protein is not predicted by "standard", commonly used bioinformatics analysis (e.g. BLASTP/PROSITE). Multiple gene knockouts of *psop12* have resulted in no detectable phenotype in blood-, sexual-, liver or pre-erythrocytic stages, suggesting that the protein could have a non-essential function within the parasite lifecycle [29,RMgmDB]. The PSOP12 protein is encoded by a 2.3 kb gene with a single 135 bp intron, located on chromosome 11, giving rise to a protein 712 amino acids in length with a projected molecular weight of 85 kDa.

Here we describe the design and evaluation of a potential TBV targeting *P. berghei* PSOP12 based on the baculovirus dual expression system (BDES-PbPSOP12-spider), enabling expression of antigens on the surface of viral particles and within infected mammalian cells [31–33]. We show that BDES-PbPSOP12-spider elicits effective PSOP12 specific humoural immune responses in mice, and confers significant transmission-blocking activity, assessed directly in vivo in the direct feeding assay (DFA), and ex vivo in the standard membrane feeding assay (SMFA). We demonstrate, using serum obtained post-immunization, and by generation of a transgenic GFP-reporter parasite, that PSOP12 is expressed exclusively in gametocytes, gametes and ookinetes. Our data suggests that PSOP12 in *Plasmodium* spp. could be a promising new TBV target candidate.

2. Materials and methods

2.1. General parasite maintenance

General parasite maintenance was carried out as described in [34]. Briefly, *P. berghei* ANKA 2.34 parasites were maintained in 6–8 week old female Tuck Ordinary (TO) mice (Harlan) by serial mechanical passage (up to a maximum of eight passages). If required, hyper-reticulosis was induced three days before infection by treating mice with intraperitoneally (i.p.) with 200 μl phenylhydrazinium chloride (PH; 6 mg/ml in PBS; Pro-Labo UK). Mice were infected i.p. and infections were monitored using Giemsa-stained tail blood smears as described previously [34].

2.2. Design and construction of recombinant baculovirus

To examine the ability of PbPSOP12 to initiate a transmission-blocking response, the recombinant baculovirus BDES-PbPSOP12-spider (Fig. 1) was generated as follows. The DNA sequence corresponding Ile_{294}–Val_{721} of *PbPSOP12* was used to generate BDES-PbPSOP12. To remove potential *N*-glycosylation sites, asparagines at amino acids 337, 608 and 700 were substituted with glutamine. The final insert was codon-optimized both for mammalian and AcNPV expression and synthesized by GenScript Corp (Piscataway, NJ, USA). The codon-optimized *PbPSOP12* gene was cloned into *EcoRI*/*XmaI* sites of pENTR-D-sPfCSP2-G2-sWPRE [35] to construct pENTR-D-sPb499-G2-sWPRE. The baculovirus transfer vector, pFast-polh-EGFP-p10-hDAF-VSV-G2-Piggy-D-sPb499-G2-sWPRE(R), was generated by incubation of pFast-polh-EGFP-p10-hDAF-VSV-G2-Piggy-RfA(R) and pENTR-D-sPb499-G2-sWPRE in the presence of LR Clonase (Invitrogen Life Technologies) following the manufacturer's instructions. The resulting pFast-polh-EGFP-p10-hDAF-VSV-G2-Piggy-D-sPb499-G2-sWPRE(R) contained three gene cassettes encoding; (1) the *PbPSOP12* gene under the control of the CMV-polyhedrin dual promoter, (2) the human decay-accelerating factor (hDAF) gene under the control of the p10 promoter, and (3) the *egfp* gene under the control of the polyhedrin promoter. The recombinant bacmid was generated by Tn7-mediated transposition of the gene cassettes in pFast-polh-EGFP-p10-hDAF-VSV-G2-Piggy-D-sPb499-G2-sWPRE(R) using the Bac-to-Bac system (Invitrogen Life Technologies), according to manufacturer's instructions. To generate BDES-PbPSOP12-spider, Sf9 cells were transfected with the appropriate bacmids (2.0 μg/well) or infected with infectious cell culture supernatants. Cell culture supernatants were collected and budded virus titres were determined using a TCID50 end-point dilution assay [40]. Virus infection was determined by monitoring EGFP expression with fluorescence microscopy.

As a non-isotypic negative control, recombinant baculovirus BDES-GL3-spider harbouring the luciferase gene (from *Photinus pyralis*) under the control of the CMV promoter was generated.

2.3. Western blotting

Protein samples from purified BDES viral particles (2.5×10^6 pfu) were separated by 8 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to an Immobilon-*P* transfer membrane (Merck Millipore), and then probed either with anti-FLAG M2 mAb with 1:2000 dilution (Sigma-Aldrich, Tokyo, Japan) or anti-CD55 (hDAF) mAb with 1:1000 dilution (Merck Millipore, Temecula, CA). Bound antibodies were subsequently detected by electrochemiluminescence (GE Healthcare, Waukesha, WI) as described previously [35].

2.4. Immunization regime

BALB/c (inbred) mice (Harlan, UK) were immunized four times at three-week intervals with 5×10^7 PFU of BDES-PbPSOP12-spider intramuscularly (i.m.). As a comparative negative control, mice were immunized with 5×10^7 PFU of BDES-GL3-spider i.m. Sera were collected prior to each injection/boost to evaluate anti-PbPSOP12 response. Final bleeds were taken two weeks after the final boost. Immunized mice were used to assess transmission-blocking responses by the direct feeding assay (DFA) as described below, following which, serum was collected by terminal anaesthesia and cardiac puncture. Serum was subsequently used in the SMFA to assess dose responsive transmission-blocking efficacy, whilst indirect fluorescent assay (IFA) was used to indicate the ability of generated anti-BDES-PbPSOP12-spider polyclonal serum to recognize the surface of the parasite.

2.5. Direct feeding assay (DFA)

Two individual cohorts of mice ($n = 5$) were immunized i.m with BEDS-PbPSOP12-spider or BDES-GL3-spider (negative control) as described above. Eight days following final boost, all mice were PH treated, and three days later infected with 10^6 iRBC with *P. berghei* ANKA 2.34. Three days post-infection, pots of starved *An. stephensi* mosquitoes (SDA 500 strain) were allowed to feed on the anesthetized infected mice. >50 mosquitoes were fed per mouse. Twenty four hours post-feed, mosquitoes were briefly anesthetized with CO_2 and unfeds removed. Mosquitoes were then maintained on 8% (w/v)) fructose, 0.05% (w/v) *p*-aminobenzoic acid at 19–22 °C and 50–80% relative humidity. Day 10 post-feeding, mosquito midguts were dissected and oocyst intensity and prevalence recorded. Reduction in intensity and prevalence in BEDS-PSOP12-spider immunized mice were calculated with respect to the BEDS-GL3-spider immunized controls.

2.6. Standard membrane feeding assay (SMFA)

An. stephensi (SDA 500 strain) were starved for 24 h and then fed on heparanized *P. berghei* infected blood using standard membrane feeding methods [34]. For each feed, 400 μl of *P. berghei* 2.34 infected blood containing asexual and sexual stages of the parasite was mixed with 200 μl of PBS containing anti-BDES-PbPSOP12-spider serum or anti-BEDS-GL3-spider serum (negative control) to yield final serum dilutions of 1:5, 1:10 and 1:50. Mosquitoes were treated and maintained as described above.

2.7. Construction of transgenic PbPSOP12-GFP

To examine the expression and localization of PbPSOP12, the *Pb*PSOP12-GFP transgenic line was created, introducing a C-terminal GFP tag to the native by single homologous recombination. The targeting construct *pPbPSOP12-GFP* was constructed using the backbone of the EGFP-tagging vector p277 [36]. The terminal 1351 bp of the *psop12* gene (PBANKA_111340) was amplified by PCR, using the oligos PS1 (5′-GGTACCGGATATTTCAAAAATCAGAGGATATTATATATCAATTATTTC-3′) and PS2 (5-′GGGCCCAACGAATGTGTAGGTAATTTCGTATAGGATG-3′), with flanking *KpnI* and *ApaI* sites introduced (underlined) to the amplicon. The resulting fragment was cloned in frame into the *ApaI*/*KpnI* site of p277, resulting in *pPbPSOP12-GFP*. For transfection, this construct was linearized at a naturally occurring *BclI* site within the *psop12* sequence (Fig. 4A).

Parasites were transfected using the Nucleofector device (Amaxa Biosystems) as described previously [36]. Integration of the DNA constructs into the chromosome was confirmed by PCR flanking a region upstream from the 5′ integration site into the EGFP sequence (oligo PS3; 5′-GCAGGGAAAAATTCACCTTCTTCGTAAC-3; oligo PS4; 5′-ACGCTGAACTTGTGGCCG-3′. Fig. 4 Panel B). Oligos against the *Pbs25* gene (PBANKA_0051500) were used as positive control (oligos: 5′-CAACTTAGCATAAATATAATAATGCGAAAGTTACCGTGG-3′, 5′-CCATCTTTACAATCACATTTATAAATTCCATC-3′). GFP expression in transfected, drug resistant parasites was confirmed by fluorescence microscopy. Two independent clones were obtained from two independent transfections, demonstrating identical phenotypes and GFP expression.

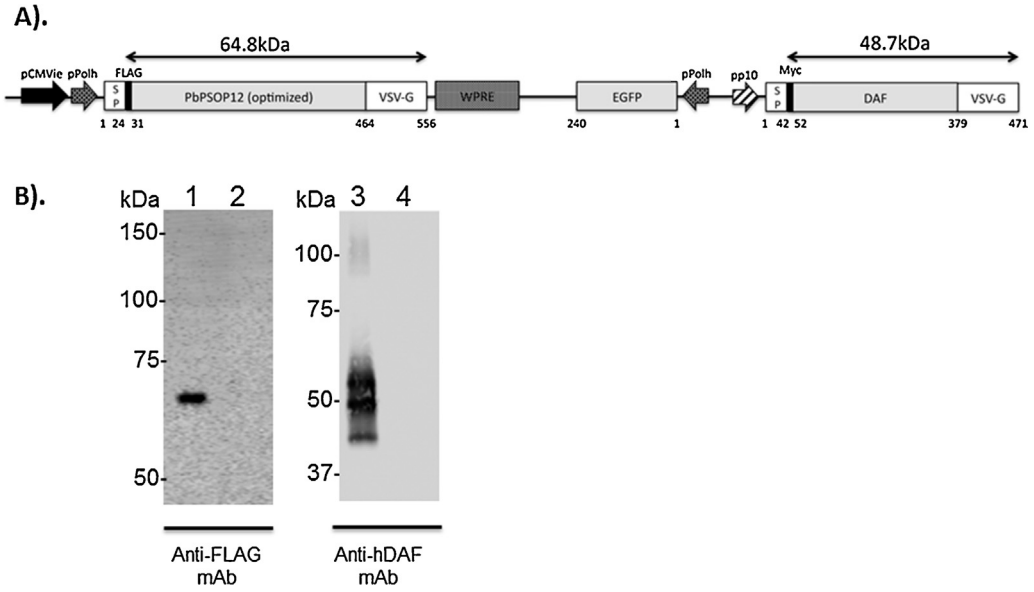

Fig. 1. Design of BDES-PbPSOP12-spider. (A) Schematic representation of BDES-PbPSOP12-spider. BDES-PbPSOP12-spider consists of the gene cassette encoding amino acids Ile_{294} to Val_{721} of PbPSOP12 fused to the DNA sequence corresponding to Phe_{421}–Arg_{511} of the VSV-G protein and the WPRE, and hDAF fused to VSV-G. SP = gp64 signal sequence. FLAG = FLAG epitope tag. Myc = Myc epitope tag. pCMVie = CMV immediate early enhancer promoter. pPolh = polyhedron promoter. pp10 = p10 baculoviral promoter. (B) Expression of PbPSOP12 by BDES-PbPSOP12-spider. Expression of PbPSOP12 and hDAF in purified viral particles was confirmed by western blotting with anti-FLAG mAb (lanes 1 and 2) and anti-hDAF mAb (lanes 3 and 4), respectively. BDES-PbPSOP12-spider was probed in lanes 1 and 3. BDES-GL3-spider (lanes 2 and 4) was used as negative control.

2.8. Immunofluorescence assay (IFA)

To confirm the expression of the PbPSOP12-GFP tagged fusion protein in our transgenic line, fluorescent parasites were visualized live. IFAs were also performed against multiple lifecycle stages (asexual parasites, gametocytes, gametes and ookinetes) using an anti-GFP rabbit IgG at 1:200 dilution (Molecular Probes, A11122) as primary antibody, and Alexa Fluor 488-labelled goat anti-rabbit IgG (Molecular Probes A11008) at 1:500 as the secondary antibody.

To assess the ability of our generated BEDS-PbPSOP12-spider antiserum to bind to the surface of the parasite, IFAs were performed against (wild-type) *P. berghei* 2.34 asexual parasites, gametocytes, gametes and ookinetes using pooled anti-BEDS-PbPSOP12-spider polyclonal serum (harvested two weeks post final bleed) at 1:200 as primary antibody, and Alexa Fluor 488-labelled goat anti-mouse IgG (Molecular Probes A11001) at 1:500 as the secondary antibody. Anti-BEDS-GL3-spider polyclonal serum was used as the appropriate negative control. Ookinetes were cultured as described in [37] and IFAs were performed as described previously [36].

2.9. Ethical statement

All procedures were performed with accordance with the terms of the UK Animals (Scientific Procedures) Act (PPL 70/7185) and were approved by the Imperial College Ethical Review Committee. The Office of Laboratory Animal Welfare (OLAW) Assurance for Imperial College London covers all Public Health Service (PHS) supported activities involving live vertebrates in the US (no. A5634-01).

2.10. Statistical analysis

Statistical analysis was performed with Graphpad Prism Software (Graphpad Software Inc.). For the DFA and SMFA, significant differences in oocyst intensity were assessed using the Mann–Whitney-*U* test ($P < 0.05$), whereas Fisher's exact probability test was used to assess differences in infection prevalence ($P < 0.05$).

3. Results

3.1. Construction of baculovirus based PSOP12 vaccine

We have previously developed a BDES-Pvs25-gp64 vaccine that displays the transmission-blocking immunogen Pvs25 on the surface of the viral envelope [31]. Immunization with this vaccine induced strong humoural immune responses with high anti-Pvs25 titres and TB activity [31]. To further improve the utility of BDES as a vaccine vector platform, the gene cassette encoding hDAF fused to the C-terminal region of G protein of vesicular stomatitis virus (VSV-G) under the control of the p10 promoter was introduced into the BDES. In addition, the gene cassette encoding EGFP under the control of the polyhedrin promoter was introduced to facilitate convenient virus titration. Using the resulting BDES (named BDES-spider), BDES-PbPSOP12-spider, harbouring the gene cassette encoding Ile_{294}–Val_{721} of PbPSOP12 fused to VSV-G and Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) under the control of a dual promoter consisting of the CMV-polyhedrin dual promoter was constructed (Fig. 1A). The C-terminal region of VSV-G comprises the ectodomain, and the transmembrane and cytoplasmic domains of VSV-G, which function to display foreign polypeptides on the baculovirus envelope [31]. BDES-GL3-spider harbouring the luciferase gene under the control of the CMV promoter and the translational enhancer element SP163 was constructed to use as a negative control for preceding experiments.

3.2. PbPSOP12-VSV-G fusion protein expression on baculoviral particles

To examine the expression of recombinant PbPSOP12 on the surface of the viral envelope, BDES-PbPSOP12-spider viral particles were purified and analysed by western blotting using an anti-FLAG mAb, which recognizes a linear epitope within the N-terminal tag of the construct (Fig. 1B). A single band with relative molecular mass (*Mr*) of 65 kDa was observed in BDES-PbPSOP12-spider,

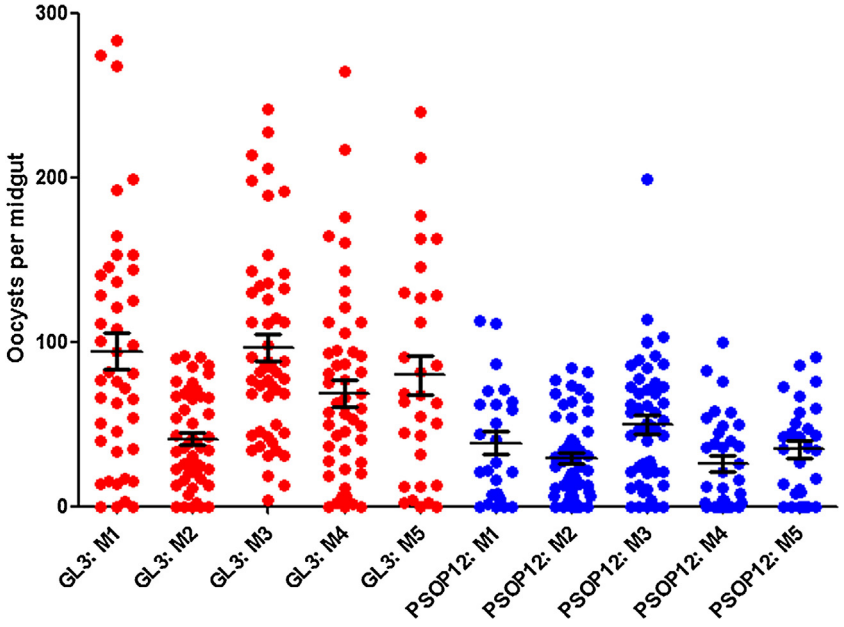

Fig. 2. Assessment of *in vivo* transmission-blocking TB efficacy by DFA in immunized mice. Two groups of five mice were immunized with either BDES-PbPSOP12-spider or BDES-GL3-spider. Each group was challenged with WT *P. berghei* 2.34, and determination of transmission blockade was performed by direct gametocyte feed. Individual data points represent the number of oocysts found in individual mosquitoes 12 days post feed. Horizontal bars indicate mean intensity of infection, whilst error bars indicate S.E.M. within individual samples.

corresponding to the predicted *Mr* 64.8 kDa of the PbPSOP12-VSV-G fusion protein (lane 1). No corresponding band was seen in BDES-GL3-spider (lane 2) because of lack of the FLAG epitope tag gene.

Baculovirus vector is inactivated by human complement *in vivo* [46], which is a potent primary barrier to *in vivo* application of baculovirus vectors. To protect BDES against the complement-mediated inactivation, hDAF was used to shield viral envelope. To confirm expression, a western blot was performed against purified viral particles using an anti-DAF mAb. A triplet band with *Mr* 55, 49 and 43 kDa was observed in BDES-PbPSOP12-spider (lane 3), which may be due to post-translational modification. No band was seen in BDES-GL3-spider because of lack of the hDAF gene (lane 4).

3.3. Immunization with BDES-PbPSOP12 induces PbPSOP12-specific antibody responses

To confirm the induction of PbPSOP12-specific antibodies to native protein on the surface of the sexual stages of the parasite by immunization with BDES-PbPSOP12-spider, we performed IFA on WT *P. berghei* 2.34, using pooled serum generated from mice immunized with BDES-PbPSOP12-spider. Surface staining on male and female gametes and ookinetes was observed (Fig. 4B), confirming the ability of our generated serum to recognize native PbPSOP12 on the surface of the sexual/ookinete stages of the parasite. Staining was not observed in non-permeablized gametocytes, suggesting a lack of surface localization in non-activated RBCs. Anti-BEDS-GL3-spider polyclonal serum, used as negative control, resulted in no detectable staining in the blood stage, or sexual stages of the parasite whether live or fixed, or permeablized or non-permeablized.

3.4. Evaluation of in vivo transmission-blocking activity (DFA)

For examination of transmission-blockade *in vivo*, mice were infected with *P. berghei* ANKA 2.34, following which *An. stephensi* mosquitoes were allowed to feed directly on the immunized and challenged hosts (Fig. 2, Table 1) three days-post infection. Mosquitoes that fed on the 5 control mice displayed an average intensity of 76.2 oocysts/midgut, whereas following i.m. immunization with BDES-PbPSOP12-spider, mean intensity was reduced to 35.7 oocysts/midgut, a reduction ($P < 0.005$) of 53.1%. Correspondingly, mean infection prevalence was reduced from 94.1% to 83.8%, a modest, but significant reduction ($P < 0.005$).

3.5. Evaluation of in vitro transmission-blocking activity (SMFA)

Following i.m. immunization with BDES-PbPSOP12-spider, pooled serum harvested from mice (16 days after final boost) profoundly reduced the intensity and prevalence of oocyst infection on the mosquito midgut in comparison with BDES-GL3-spider isotypic control serum (Fig. 3, Table 2). Inhibition was dose dependent. Immune serum, diluted 1:5, inhibited parasite intensity by 76.4% and reduced prevalence by 47.2%. At a dilution of 1 in 10, intensity was reduced by 53.9% and prevalence by 18.9%. Conversely, at the lowest dilution tested (1 in 50), the presence of serum resulted in no significant transmission-blocking effect, with a small non-significant increase (5.5%) in infection prevalence recorded. In contrast to anti-BDES-PbPSOP12-spider serum, the presence of anti-BDES-GL3-spider sera resulted in parasite intensities and prevalence of infection that were not significantly different from each other, irrespective of dilution.

3.6. Expression and localization of PbPSOP12

To additionally examine the localization of PbPSOP12 on the sexual stages of the parasite, and ascertain its accessibility to attack by antibodies induced by the host immune system, we utilized targeted-single homologous recombination to generate a transgenic *P. berghei* parasite, expressing the endogenous PbPSOP12 protein with a C-terminal EGFP fusion tag (Fig. 4A). Successful integration following drug selection was confirmed by PCR using the specific primers PS3 and PS4, giving rise to a single band of 1619 kb (comprising of 143 bp of the upstream region, 1359 bp of the *psop12* locus and 117 bp of the e*gfp* ORF), spanning a region upstream from the *psop12* locus, the recombination site within the

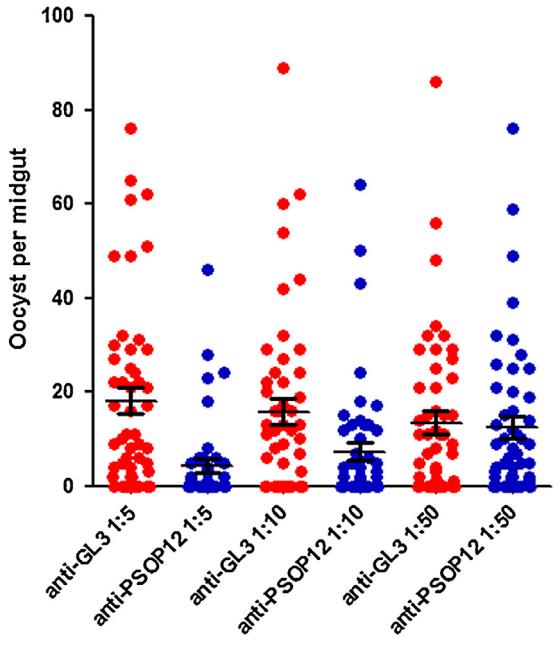

Fig. 3. Determination of *in vitro* transmission-blocking efficacy of anti-*P. berghei Pb*PSOP12 serum using the SMFA. *P. berghei* WT 2.34 infected blood was mixed with anti-PbPSOP12-spider mouse sera or mouse BEDS-GL3-spider sera (negative control) at final dilutions of 1 in 5, 1 in 10 and 1 in 50. Individual data points represent the number of oocysts found in individual mosquitoes 12 days post feed. Horizontal bars indicate mean intensity of infection, whilst error bars indicate S.E.M. within individual samples.

gene itself, and the region encoding gfp (Fig. 4C). The C-terminal EGFP tag caused no obvious growth defect in blood or sexual stages, and did not interfere with parasite transmission through *An. stephensi* mosquitoes. Live fluorescence microscopy showed PbPSOP12-EGFP staining in micro- and macrogametocytes, activated male and female gametes, and ookinetes (Fig. 4B). Staining was not observed in the blood stages of the parasite lifecycle or in later mosquito stages. This staining pattern was observed in all transgenic parasites examined over three independent experiments by fluorescence microscopy following drug selection with pyrimethamine. In all stages where expression was observed much of the fluorescence signal emanated from the cell periphery, suggesting localization to the plasmalemma, as predicted previously *in silico* [27]. This was further confirmed by IFA on fixed parasites performed with anti-EGFP mAb under non-permeablizing conditions, which demonstrated surface staining in the gametes and ookinetes. Identical surface staining was noted in transgenic PbPSOP12-EGFP parasites, and in WT *P. berghei* parasites stained with anti-BDES-PbPSOP12-spider serum, confirming the localization of PbPSOP12 on the surface of the sexual/ookinete stages of the parasite, whilst suggesting that the presence of the EGFP-fusion protein in the transgenic line does not alter the native localization of the protein. Staining in the zygote was not observed.

4. Discussion

Transmission-blocking vaccines aim to reduce the burden of malaria by preventing transmission of *Plasmodium* from humans, carrying the sexually active forms of the parasite within their peripheral blood, to mosquitoes. By targeting molecules on the parasite surface, it is possible to kill parasites or disrupt their development within the anopheline vector, preventing onwards transmission. Previous studies on multiple antigens (most notably P25, P28, P48/45, P230 and HAP2) have consistently demonstrated the utility of this approach to malarial control. Furthermore, recent lab-based "bottle" studies have demonstrated that anti-malarial transmission-blocking interventions can be capable of eliminating *Plasmodium* from vertebrates in the absence of other interventions [39]. Despite numerous studies investigating the utility of a

Table 1
In vivo evaluation of transmission-blocking effect of BEDS-PbPSOP12-spider. Changes in intensity (arithmetic mean number of oocyts per midgut) and prevalence in groups of mice immunized with BEDS-PbPSOP12-spider were calculated with respect to the appropriate, grouped BEDS-GL3-spider immunized mice.

	GL3: M1	*GL3: M2*	*GL3: M3*	*GL3: M4*	*GL3: M5*	*PSOP12: M1*	*PSOP12: M2*	*PSOP12: M3*	*PSOP12: M4*	*PSOP12: M5*
No. mosquitoes	43	50	50	50	30	25	55	50	33	28
No. infected	40	45	50	47	28	21	50	45	25	22
No. non-infected	3	5	0	3	2	4	5	5	8	6
Infection prevalence (%)	93	90	100	94	93	84	91	90	75.8	78.6
Oocyst Intensity	94.5	41.1	96.7	68.3	79.8	38.5	29.5	46.8	26.3	34.6
S.E.M	11.3	3.9	8.4	8.3	12.2	7.0	3.3	5.4	4.9	5.4
Mean prevalence (%)	94.1					83.8				
Mean intensity	76.2					35.7				
Inhibition in prevalence	–					**10.9**[a]				
Inhibition in intensity (%)	–					53.1[b]				

[a] $P < 0.05$, Fisher's exact test.
[b] $P < 0.05$, Mann–Whitney *U* test.

Table 2
Evaluation of transmission-blocking effect of anti-*P. berghei* PSOP12 serum by SMFA. Change in intensity (mean number of oocyts per midgut) and prevalence with dilution of BEDS-derived anti-PbPSOP12 mouse sera at 1 in 5, 1 in 10 and 1 in 50 were calculated with respect to appropriate serum derived from BEDS-GL3-spider controls at the relevant concentration.

	GL3: 1:5	*PSOP12: 1:5*	*GL3: 1:10*	*PSOP12: 1:10*	*GL3: 1:50*	*PSOP12: 1:50*
No. mosquitoes	50	45	50	50	50	50
No. infected	40	19	33	27	36	38
No. non-infected	10	26	17	23	14	12
Infection prevalence (%)	80	42	66	54	72	76
Oocyst intensity	18.1	4.3	15.7	7.24	13.4	12.5
S.E.M	2.8	1.4	2.7	1.9	2.4	2.3
Inhibition in prevalence (%)	47.2[a]		18.9		−5.5	
Inhibition in intensity (%)	76.4[b]		53.9[b]		6.7	

[a] $P < 0.05$, Fisher's exact test.
[b] $P < 0.05$, Mann–Whitney *U* test.

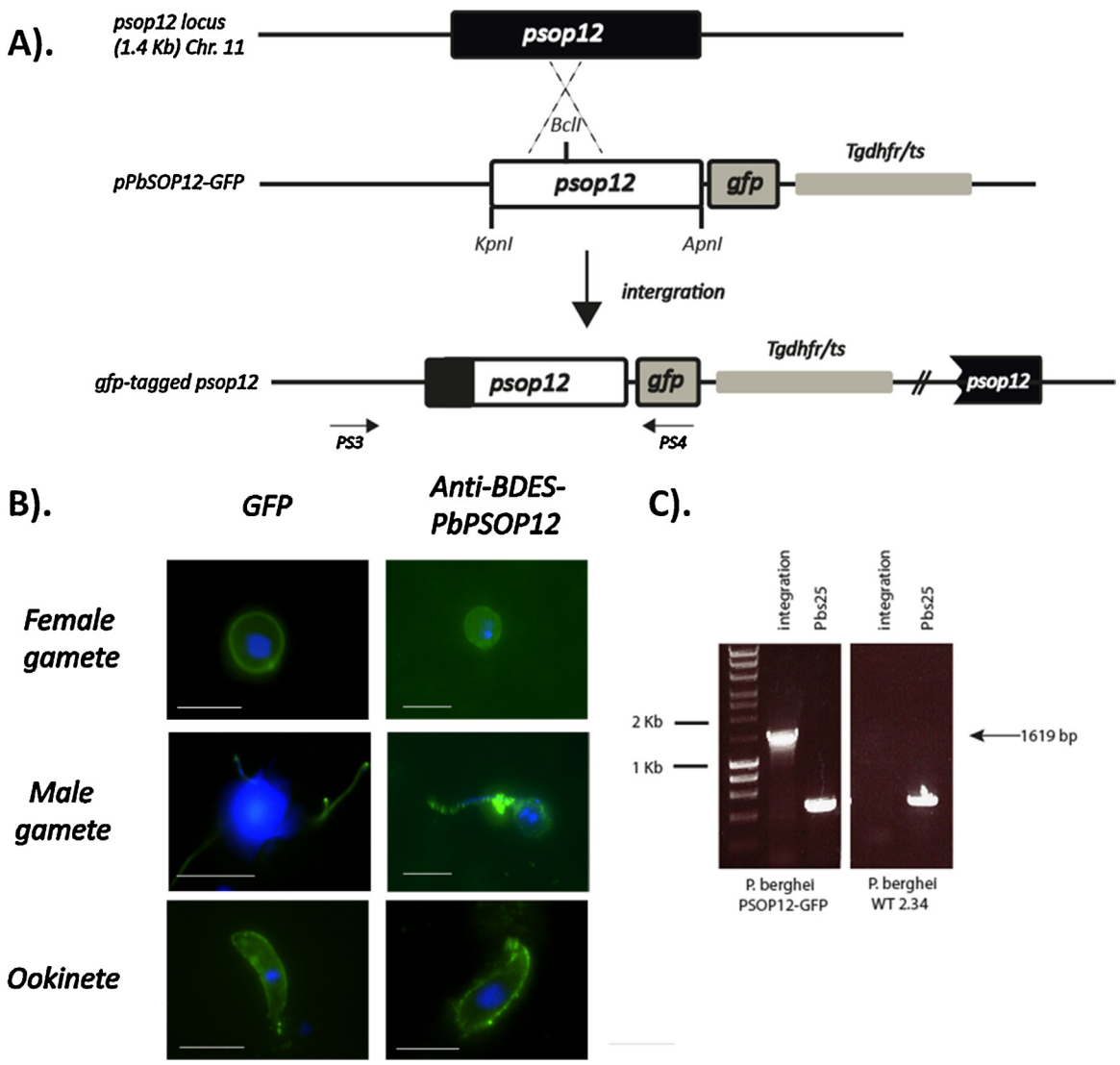

Fig. 4. GFP tagging of the endogenous *psop12* locus, and localization in the sexual stages of *P. berghei*. (A) Schematic showing the *psop12* locus, the targeting vector pPbSOP12-GFP, and the insertion of an e*gfp* sequence in-frame within the endogenous locus. (B). Fluorescence micrographs of live parasites expressing PbPSOP12-GFP, or fixed, non-permablized parasites probed with anti-BDES-PbPSOP12-spider serum. Each panel shows an overlay of GFP fluorescence (green) and DNA labelled with Hoechst (blue). White scale bars indicate 10 μm. IFA with anti-BDES-GL3-spider (control) serum resulted in no observable staining. (C) PCR confirmation of integration of e*gfp* into the *psop12* locus. Oligonucleotides PS3 and PS4 were used to detect integration, *pbs25* oligos were used as positive controls. *P. berghei* WT 2.34 gDNA was used as a negative control for integration.

number of TBV antigens over the last 40 years, there is a currently a real need for new potential TBV vaccine candidates to support the continuing development pipeline [4].

This study describes the evaluation of serum antibodies raised against baculovirus-expressed PbPSOP12 as a transmission-blocking agent. The biological significance or function of PbPSOP12 still remains to be elucidated. Multiple gene knockouts [29,RMgmDB] of the *psop12* locus resulted in no detectable phenotype, indicating a non-essential role within the parasite lifecycle. Both in silico predictions [27,29,30], and the localization carried out within this study indicate that PbPSOP12 is located on the surface of the sexual stages of the parasite (namely the male and female gametes, and ookinetes), and as such is accessible for antibody-mediated attack as part of a transmission-blocking strategy. This criteria is logically vital for the ability of an antigen to induce a transmission-blocking response and the experimentation described here illustrates that surface localization should be considered a vital marker to assess if a polypeptide should be considered a putative TBV target. Screens for novel vaccine candidates based on knock-outs or phenotypic analysis (e.g. [29]) can undoubtedly yield interesting and valuable information regarding cell biology, but surface localization on the sexual stages of the target should be considered a key parameter for the triage and selection of novel transmission-blocking candidates. As further demonstration of this concept, the current lead TBV antigen, P25 is currently considered to be “non-essential”, and its knockout results in no detectable phenotype (RMgmDB).

An additional essential attribute for the selection of new TBV antigens is the ability to induce a strong immune response following immunization with the appropriate recombinant protein/immunogen. Within the scope of this study we achieve this by utilizing the baculovirus dual expression system [31]. We demonstrate that sera harvested from mice immunized with BDES-PbPSOP12-spider is capable of specifically recognizing native PbPSOP12 on the surface of the sexual stages of the parasite, indicating the potential utility of BDES-PbPSOP12 as an efficacious TBV,

and the ability of the BDES system to express vaccine candidates, even those with 6-Cys domains.

Numerous protein expression/adjuvant/delivery systems have been tested over previous years for their ability to induce TB responses with varying success. Specifically, when dealing with plasmodial proteins, the A+T rich nature of the genome [42] can lead to difficulties in producing recombinant protein containing such native epitopes (48/45). "Standard" immunization regimes, using recombinant protein, either alone or in combination with adjuvants (e.g. Freund's, aluminium hydroxide, cholera toxin (CT) [40,41]) all indicate that immunized proteins requires linear or conformation dependent epitopes, and a strong adjuvant to induce transmission-blocking antibodies. Use of commonly utilized adjuvants to initiate potent immune responses can often have undesirable side effects and questionable safety profiles (CT) [43]. In contrast to this, the BDES circumvents standard protein expression issues by initiating viral expression of polypeptides, and has been successfully used to initiate immune responses and induce transmission-blocking activity against a wide range of *Plasmodium* proteins in the past [31–33,35]. It may additionally offer an attractive immunization method as it exhibits low cytotoxicity and is incapable of replication in mammalian cells. [31]. The value of using a system similar to this in a suitable small-scale animal model for the in vivo assessment of vaccine-induced transmission-blocking functional responses is demonstrated by this study. Put simply, it is much cheaper, easier, and ethically less problematic to identify and triage new anti-malarial vaccine candidates in a rodent malarial model before proceeding to validation in expensive and ethically complex human clinical trials. By combining the convenient baculovirus dual expression system with the highly tractable [37] *P. berghei* model, we have a powerful tool to identify, triage and examine new potential TBV candidates, with the possibility of adding comparatively simple analysis of cell biology in rodent malaria parasites if desired. Despite this undoubted application in animal models, the use of baculoviral delivery systems still requires validation in human trials. A recently published study has demonstrated that baculovirus dual expression based vaccines are safe and well tolerated, with acceptable reactogenicity and systemic toxicity in a primate model [35]. It would be advantageous to perform similar studies in the future to examine fully the possibility of offering an alternative to current vaccine delivery platforms for clinical trials.

Within the scope of this study, the BDES-PbPSOP12-spider vaccine initiated modest, yet significant, transmission blockade *in vivo* following active immunization (53.1% reduction in intensity, 10.9% reduction in prevalence). Higher transmission blockade was seen with *in vitro* experimentation in the SMFA (up to a 76.4% reduction in intensity and a 47.2% reduction in prevalence). It remains to be seen if increased transmission blocking potency could be initiated using this antigen with different baculoviral expression constructs, delivery systems based on other viral vectors (e.g. adenovirus [44]) or standard recombinant protein/adjuvant immunization methods [21]. PSOP12 is the third member of the 6-Cys protein family, with P48/45 and P230, to demonstrate the ability to initiate an anti-malarial transmission blocking response. There are currently 14 putative members of the 6-Cys family identified in *Plasmodium*, with 10 initially identified [19], and a further 4 characterized following in-depth structural studies [28,45]. Family members are often located on the parasite surface, suggesting a function in cell-cell interactions (e.g. the 6-Cys proteins P52, P36, sequestin and B9 play vital roles related to infection of the liver by the parasite [28]). Five family members have previously been characterized as expressed in the sexual stages of the parasite, and three of the proteins that are expressed in the gamete function in the recognition and adhesion between male and female gametes [19]. This study supports previous in-depth studies highlighting the potential of members of the 6-Cys to act as TBV vaccine candidates, and suggests that continued analysis of family members could be advantageous.

5. Conclusions

In this study we design, construct and evaluate a novel TBV targeting the 6-Cys protein family member PSOP12 in *P. berghei*. Expression of the protein in the baculovirus dual expression system, followed by immunization of mice induces specific humoural immune response. The resulting serum recognizes the surface of the sexual and ookinete stages of the parasite in a manner consistent with observed expression of the PSOP12 protein. A range of TB assays demonstrate significant TB efficacy both in vivo and in vitro using the sera raised. As such, our data indicates that PSOP12 in *Plasmodium* spp. could be a promising new TBV target candidate, and that further experimentation to examine the protein within human malaria parasites could be advantageous.

Conflict of interest statement

The authors are not aware of any conflicts of interest arising from this work.

Acknowledgements

A.M.B. thanks MVI-PATH (USA), MMV (Geneva), Transmalbloc and the Fraunhofer Institute for funding K.A.S. We gratefully acknowledge Dr. Mark. Wass (University of Kent) for Bioinformatics assistance, Kentaro Takagi and Yuki Teramura for plasmid construction, and Mark Tunnicliff for mosquito production. This work was supported in part by MVI-PATH (USA) for K.A.S. & L.U.M., MMV for S.E.Z., the Bill and Melinda Gates Foundation (OPP1043501) for M.J.D., Grants-in-Aid for Scientific Research (B) (25305007) from the Japan Society for the Promotion of Science (JSPS) and Japan-UK Joint Research Projects of JSPS Bilateral Programs (14039901-000143) to S.Y

References

[1] WHO. World Malaria Report. World Health Organization; 2013.
[2] Griffin JT, et al. Estimates of the changing age-burden of *Plasmodium falciparum* malaria disease in sub-Saharan Africa. Nat Commun 2014;5:3136, http://dx.doi.org/10.1038/ncomms4136.
[3] Gatton ML, et al. The importance of mosquito behavioural adaptations to malaria control in Africa. Evolution 2013;67(4):1218–30.
[4] The malERA Consultative Group on Vaccines A. Research agenda for malaria eradication: vaccines. PLoS Med 2011;8(1.):E1000398.
[5] Grotendorst CA, Carter R, Rosenberg R, Koontz LC. Complement effects on the infectivity of *Plasmodium gallinaceum* to *Aedes aegypti* I. Resistance of zygotes to the alternative pathway of complement mosquitoes. J Immunol 1986;136(11):4270–4.
[6] Kaslow DC, Bathurst IC, Barr PJ. Malaria transmission-blocking vaccines. Trends Biotechnol 1992;10(11):388–91.
[7] Vermeulen AN, Ponnudurai T, Beckers PJ, Verhave JP, Smits MA, Meuwissen JH. Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. J Exp Med 1985;162(5):1460–76.
[8] Ranawaka G, Fleck SL, Alejo-Blanco A, Sinden R. E. Characterisation of the modes of action of anti Pbs21 malaria transmission-blocking immunity upon ookinete to oocyst differentiation in vivo. Parasitology 1994;109(4):403–41.
[9] Carter R, Mendis KN, Miller LH, Molineaux L, Saul A. Malaria transmission blocking vaccines: how can their development be supported? Nat Med 2000;6:241–4.
[10] Kaslow DC. Transmission-blocking vaccines. Chem Immunol 2002;80:287–307.
[11] van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JA, Dodemont HJ, et al. A central role for P48/45 in malaria parasite male gamete fertility. Cell 2001;104:153–64.
[12] Roeffen W, Lensen T, Mulder B, Teelen K, Sauerwein R, Van Druten J, et al. A comparison of transmission-blocking activity with reactivity in a *Plasmodium falciparum* 48/45-kD molecule-specific competition enzyme-linked immunosorbent assay. Am J Trop Med Hyg 1995;52:60–5.

[13] Bousema JT, Drakeley CJ, Kihonda J, Hendriks JC, Akim NI, Roeffen W, Sauerwein RW. A longitudinal study of immune responses to *Plasmodium falciparum* sexual stage antigens in Tanzanian adults. Parasite Immunol 2007;29:306–17.
[14] Roeffen W, Mulder B, Teelen K, Bolmer M, Eling W, et al. Association between anti-Pfs48/45 reactivity and *P. falciparum* transmission-blocking activity in sera from Cameroon. Parasite Immunol 1996;18:103–9.
[15] Outchkourov NS, Roeffen W, Kaan A, Jansen J, Luty A, et al. Correctly folded Pfs48/45 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in mice. Proc Natl Acad Sci USA 2008;105:4301–5.
[16] Williamson KC, Keister DB, Muratova O, Kaslow DC. Recombinant Pfs230, a *Plasmodium falciparum* gametocyte protein, induces antisera that reduce the infectivity of *Plasmodium falciparum* to mosquitoes. Mol Biochem Parasitol 1995;75:33–42.
[17] Healer J, McGuinness D, Hopcroft P, Haley S, Carter R, et al. Complement-mediated lysis of *Plasmodium falciparum* gametes by malaria-immune human sera is associated with antibodies to the gamete surface antigen Pfs230. Infect Immun 1997;65:3017–23.
[18] Eksi S, Czesny B, van Gemert GJ, Sauerwein RW, Eling W, et al. Malaria transmission-blocking antigen. Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production. Mol Microbiol 2006;61:991–8.
[19] van Dijk MR, van Schaijk BC, Khan SM, van Dooren MW, Ramesar J, Kaczanowski S, et al. Three members of the 6-cys protein family of *Plasmodium* play a role in gamete fertility. PLoS Pathog 2010;6(Apr (4)):e1000853.
[20] Blagborough AM, Sinden RE. *Plasmodium berghei* HAP2 induces strong malaria transmission-blocking immunity *in vivo* and *in vitro*. Vaccine 2009;27(Aug (38)):5187–94.
[21] Miura K, Takashima E, Deng B, Tullo G, Diouf A, Moretz SE, et al. Functional comparison of *Plasmodium falciparum* transmission-blocking vaccine candidates by the standard membrane-feeding assay. Infect Immun 2013;81(Dec (12)):4377–82.
[22] Theisen M, Roeffen W, Singh SK, Andersen G, Amoah L, van de Vegte-Bolmer M, et al. A multi-stage malaria vaccine candidate targeting both transmission and asexual parasite life-cycle stages. Vaccine 2014;32(May (22)):2623–30.
[23] Vermeulen AN, Ponnudurai T, Beckers PJ, Verhave JP, Smits MA, et al. Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. J Exp Med 1985;162:1460–76.
[24] Winger LA, Tirawanchai N, Nicholas J, Carter HE, Smith JE, Sinden RE. Ookinete antigens of *Plasmodium berghei*. Appearance on the zygote surface of an Mr 21 kD determinant identified by transmission-blocking monoclonal antibodies. Parasite Immunol 1988 Mar;10(2):193–207.
[25] Liu Y, Tewari R, Ning J, Blagborough AM, Garbom S, Pei J, et al. The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in *Chlamydomonas* and *Plasmodium* gametes. Genes Dev 2008;22(Apr (8)):1051–68.
[27] Wass MN, Stanway R, Blagborough AM, Lal K, Prieto JH, Raine D, et al. Proteomic analysis of *Plasmodium* in the mosquito: progress and pitfalls. Parasitology 2012;139(Aug (9)):1131–45.
[28] Annoura T, van Schaijk BC, Ploemen IH, Sajid M, Lin JW, Vos MW, et al. Two *Plasmodium* 6-Cys family-related proteins have distinct and critical roles in liver-stage development. FASEB J 2014;28(May (5)):2158–70.
[29] Ecker A, Bushell ES, Tewari R, Sinden RE. Reverse genetics screen identifies six proteins important for malaria development in the mosquito. Mol Microbiol 2008;70(Oct (1)):209–20.
[30] Hall N, Karras M, Raine JD, Carlton JM, Kooij TW, Berriman M, et al. A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. Science 2005;307(Jan (5706)):82–6.
[31] Blagborough AM, Yoshida S, Sattabongkot J, Tsuboi T, Sinden RE. Intranasal and intramuscular immunization with baculovirus dual expression system-based Pvs25 vaccine substantially blocks *Plasmodium vivax* transmission. Vaccine 2010;28(Aug (37)):6014–20.
[32] Yoshida S, Kawasaki M, Hariguchi N, Hirota K, Matsumoto M. A baculovirus dual expression system-based malaria vaccine induces strong protection against *Plasmodium berghei* sporozoite challenge in mice. Infect Immun 2009;77(May (5)):1782–9178.
[33] Mlambo G, Kumar N, Yoshida S. Functional immunogenicity of baculovirus expressing Pfs25, a human malaria transmission-blocking vaccine candidate antigen. Vaccine 2010;28(Oct (43)):7025–9.
[34] Blagborough AM, Delves MJ, Ramakrishnan C, Lal K, Butcher G, Sinden RE. Assessing transmission blockade in *Plasmodium* spp. Methods Mol Biol 2013;923:577–600.
[35] Iyori M, Nakaya H, Inagaki K, Pichyangkul S, Yamamoto DS, Kawasaki M, et al. Protective efficacy of baculovirus dual expression system vaccine expressing *Plasmodium falciparum* circumsporozoite protein. PLoS One 2013;8(Aug (8)):e70819.
[36] Sebastian S, Brochet M, Collins MO, Schwach F, Jones ML, Goulding D, et al. A *Plasmodium* calcium-dependent protein kinase controls zygote development and transmission by translationally activating repressed mRNAs. Cell Host Microbe 2012;12(Jul (1)):9–19.
[37] Ramakrishnan C, Delves MJ, Lal K, Blagborough AM, Butcher G, Baker KW, et al. Laboratory maintenance of rodent malaria parasites. Methods Mol Biol 2013;923:51–72.
[39] Blagborough AM, Churcher TS, Upton LM, Ghani AC, Gething PW, Sinden RE. Transmission-blocking interventions eliminate malaria from laboratory populations. Nat Commun 2013;4:1812.
[40] Malkin EM, Durbin AP, Diemert DJ, Sattabongkot J, Wu Y, Miura K, et al. clinical trial of Pvs25H: a transmission blocking vaccine for *Plasmodium vivax* malaria. Vaccine 2005;23:3131–8.
[41] Stowers A, Carter R. Current developments in malaria transmission-blocking vaccines. Expert Opin Biol Ther 2001;1:619–28.
[42] Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 2002;419(Oct (6906)):498–511.
[43] Hagiwara Y, Iwasaki T, Asanuma H, Sato Y, Sata T, Aizawa C, et al. Effects of intranasal administration of cholera toxin (or *Escherichia coli* heat-labile enterotoxin) B subunits supplemented with a trace amount of the holotoxin on the brain. Vaccine 2001;8(Feb (13–14)):1652–60.
[44] Goodman AL, Blagborough AM, Biswas S, Wu Y, Hill AV, Sinden RE, et al. A viral vectored prime-boost immunization regime targeting the malaria Pfs25 antigen induces transmission-blocking activity. PLoS One 2011;6(12):e29428.
[45] Arredondo SA, Cai M, Takayama Y, MacDonald NJ, Anderson DE, Aravind L, et al. Structure of the *Plasmodium* 6-cysteine s48/45 domain. Proc Natl Acad Sci USA 2012;109(Apr (17)):6692–7, http://dx.doi.org/10.1073/pnas.1204363109.
[46] Kaikkonen MU1, Maatta AI, Ylä-Herttuala S, Airenne KJ. Screening of complement inhibitors: shielded baculoviruses increase the safety and efficacy of gene delivery. Mol Ther 2010;18(May (5)):987–92.